Angiotensin II Induces Myocyte Enhancer Factor 2– and Calcineurin/Nuclear Factor of Activated T Cell–Dependent Transcriptional Activation in Vascular Myocytes

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Abstract—It is well known that angiotensin II (Ang II) is implicated in the phenotypic modulation and hypertrophy of vascular smooth muscle cells (VSMCs). To study the mechanisms by which Ang II contributes to the pathological changes of VSMCs, we examined whether Ang II stimulated myocyte enhancer factor 2 (MEF2)- and calcineurin/nuclear factor of activated T cell (NFAT)-dependent transcriptional activation of genes in VSMCs. Ang II increased the DNA binding activity of MEF2A and its expression at the protein level. Ang II induced c-jun promoter activity, and this increase was inhibited by dominant-negative mutants of MEF2A and mitogen-activated protein kinase kinase 6 but not by calcineurin inhibitors. Ang II stimulated NFAT DNA binding activity and NFAT-dependent gene transcription, and these effects of Ang II were inhibited by calcineurin inhibitors. Furthermore, Ang II induced the promoter activity of the nonmuscle-type myosin heavy chain B gene, which we used as a marker of the dedifferentiated state of VSMCs, and this increase was inhibited by calcineurin inhibitors but not by the dominant-negative mutants of MEF2A or mitogen-activated protein kinase kinase 6. Finally, Ang II increased protein synthesis, and this increase was inhibited by infection with an adenovirus construct that expresses the dominant-negative mutant of MEF2A but not by calcineurin inhibitors. These results suggest that Ang II stimulates the MEF2- and calcineurin/NFAT-dependent pathways and that these pathways have distinct roles in VSMCs. (Circ Res. 2002;90:1004-1011.)

Key Words: angiotensin II • myocyte enhancer factor 2 • calcineurin/nuclear factor of activated T cells • vascular smooth muscle cells • atherosclerosis

Atherosclerosis results from chronic inflammation of blood vessels in which vascular endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and macrophages interact to induce pathophysiological changes in the vessel wall. In the early phase of atherosclerosis, vascular ECs express adhesion molecules. Monocytes in circulating blood attach to vascular ECs via those adhesion molecules, infiltrate the vessel wall, and become activated macrophages. These macrophages produce a variety of cytokines, which in turn promote phenotypic modulation/dedifferentiation of VSMCs and induce their migration, hypertrophy, and hyperplasia.1 Although the accumulated evidence suggests that the renin-angiotensin system is implicated in the pathogenesis of atherosclerosis,2–6 little is known regarding what kinds of intracellular signaling pathways and transcription factors are implicated in angiotensin II (Ang II)–induced progression of atherosclerosis.

Myocyte enhancer factor 2 (MEF2), related to serum response factor protein, is a family of transcription factors that consists of 4 isoforms: MEF2A, MEF2B, MEF2C, and MEF2D.7–10 The MEF2 family of transcription factors appears to cooperate with the MyoD family of transcription factors and to promote the myogenesis of skeletal muscle cells.11 MEF2C-null mice do not form a normal heart and vascular system,12,13 suggesting that MEF2 is required for the development of the cardiovascular system. Although it is not clear whether MEF2 plays critical roles in the pathogenesis of atherosclerosis, we recently reported that MEF2A was a major isoform expressed in VSMCs and that MEF2A expression, its DNA binding activity, and its transactivating function increased when serum-starved VSMCs were restimulated with serum mitogen.14 These results suggest that MEF2 may play roles in the phenotypic modulation and/or hypertrophy of VSMCs rather than in the maintenance of the differentiated state of VSMCs.

Although the molecular mechanisms of vascular hypertrophy remain unsolved, the mechanisms of cardiac hypertrophy have begun to be unveiled. Recent progress in this field has clearly shown that the Ca2+-dependent activation of a phosphatase called calcineurin is critically implicated in cardiac...
hypertrophy. When calcineurin is activated, it dephosphorylates transcription factors called nuclear factors of activated T cells (NFATs), which in turn promote nuclear translocation of NFAT. The NFAT transcription factors then cooperate with nuclear transcription factors such as GATA-4 and stimulate the transcriptional activation of various genes that are involved in cardiac hypertrophy. However, little is known regarding whether the calcineurin/NFAT-dependent pathway is implicated in the phenotypic modulation or hypertrophy of VSMCs.

The difficulty in studying the mechanisms involved in the phenotypic modulation of VSMCs is largely attributable to the lack of any established markers of the dedifferentiated state of VSMCs. Recently, it has been shown that an isoform of myosin heavy chain, known as nonmuscle-type myosin heavy chain B (NMHC-B)/SMemb, is expressed in undifferentiated VSMCs during the embryonic period and that its expression is increased in the neointima of balloon-injured arteries, suggesting that the expression of NMMHCB can be used as a marker of phenotypic modulation of VSMCs. However, it also remains to be determined whether the MEF2- and/or calcineurin/NFAT-dependent pathway is implicated in the expression of NMMHCB.

In the present study, we examined whether Ang II induced the MEF2- and calcineurin/NFAT-dependent transcriptional activation of genes in VSMCs. We also examined whether Ang II induced the expression of NMMHCB and whether the Ang II–induced NMMHCB expression was mediated by the MEF2- or calcineurin/NFAT-dependent pathway. Finally, we examined whether Ang II–induced vascular hypertrophy was mediated by the MEF2- or calcineurin/NFAT-dependent pathway.

### Materials and Methods

#### Reagents

Valsartan (Val) and cyclosporin A (CyA) were kindly supplied by Novartis Pharma AG (Basel, Switzerland), and FK506 (FK) was supplied by Fujisawa Pharmaceutical Co, Ltd (Osaka, Japan). SB203580 (SB) was purchased from Sigma Chemical Co. The anti-MEF2A antibody used for Western blot analyses and anti-NFAT3 antibody were obtained from Santa Cruz Biotechnology. Anti-hemagglutinin antibody was purchased from Roche Molecular Biochemicals.

#### Cell Culture

Rat VSMCs were cultured from rat thoracic aortas after the explant method, as previously described.19 Human VSMCs were obtained from Sanko Junyaku and maintained in DMEM containing 20% FBS.

#### Plasmids

Details of the cloning of human MEF2A wild type, MEF2AS545A (MEF2ASA), in which serine 453 was substituted with alanine, human calcineurin A 1-398 (CalA2C), which lacks the carboxy-terminal calmodulin binding domain, and MEK6S207A/T211A (MEK6A, where MEK6 is mitogen-activated protein kinase kinase 6) and MEK6S207E/T211E (MEK6EE), in which serine 207 and threonine 211 were replaced with alanine and glutamic acid, respectively, are described elsewhere (see online supplementary information). Cloning of the human c-jun promoter and human NMMHC promoter and construction of the heterologous promoter in which 3 copies of the wild-type or mutant NFAT binding site were subcloned upstream from the minimal essential promoter region of the murine c-fos gene are also described elsewhere (see online supplementary information). The carboxyl-terminal coding region of the human c-jun gene was isolated and used for Northern blot analysis as described elsewhere (see online supplementary information).

#### Transient Transfection

pRL-TK, which encodes the SeaPansy luciferase gene, was purchased from Toyo Ink and used as the internal control for the luciferase assays. With the use of lipofectAMINE (Life Technologies), rat VSMCs were transiently transfected with reporter plasmids encoding the wild-type or mutant c-jun promoter, putative NFAT binding sites upstream from the murine c-fos minimal essential promoter, or NMMHCB promoter, along with pRL-TK. Rat VSMCs were then stimulated with 10⁻⁷ mol/L Ang II for 48 to 96 hours and harvested for luciferase assays. In some experiments, cells were pretreated with 100 nmol/L Val, 1 μmol/L CyA, 1 μmol/L FK, or 10 μmol/L SB and stimulated with 10⁻⁷ mol/L Ang II. Rat VSMCs were also cotransfected with MEF2ASA, MEK6A, MEK6EE, or CalA2C in some experiments to examine the effects of these mutants on the activities of those promoters. Dual luciferase assay was performed by using a luminometer (Lumat LB 9507, Berthold).

#### Construction of a Replication-Defective Adenovirus

A replication-defective adenovirus that expresses hemagglutinin-tagged MEF2ASA (AdMEF2ASA) or CalA2C (AdCalA2C) was constructed with the use of an AdMax kit (Microbix Biosystems Inc) by the methods recommended by the manufacturer. The protein-coding region of MEF2ASA and CalA2C was ligated to the pDC516 vector and cotransfected into 293 cells along with pBHGfrtΔE1.3FLP. Recombinant adenoviruses were generated through homologous recombination, propagated in 293 cells, and, finally, purified by CsCl gradient ultracentrifugation. A recombinant adenovirus that expresses green fluorescence protein (AdGFP) was obtained from Quantum Biotechnologies.

#### Western Blot Analysis

Protein extraction for Western blot analyses was performed as previously described.20 Antibodies were used at a dilution of 1:200 except for anti-NMHC-B antibody, which was used at a dilution of 1:1000. The details of the preparation of anti-NMHC-B antibody used in the present study have been previously described.

#### Electrophoretic Mobility Shift Assays

VSMCs were serum-starved for 3 days and stimulated with 10⁻⁷ mol/L Ang II for 8 hours. Whole-cell extracts were prepared from rat VSMCs as described previously.14 Probes and competitor DNAs were double-stranded synthetic oligonucleotides that were prepared according to the sequence of the MEF2 binding site from the human c-jun promoter and the NFAT binding site from the human interleukin-2 promoter. The nucleotide sequences of the sense strand of the double-stranded oligonucleotides were as follows: for wild-type MEF2, 5'-TCAGCTATTTTTAGGCTTGACGAGCT-3'; for mutant MEF2, 5'-TCAGCTATTTTTAGCCTGTAACGAGCT-3'; for wild-type NFAT, 5'-CTACATTGATGGATTTTATTACATTAGCACGAGCT-3'; and for mutant NFAT, 5'-CTACATTGATGGATTTTATTACATTAGACGACT-3'.

The lowercase bold letters depict nucleotide substitutions to induce point mutations. Electrophoretic mobility shift assays (EMSAs) were performed as previously described.14 A highly specific anti-MEF2A antibody used for supershift assay has previously been described.

#### Northern Blot Analysis

Total RNA was extracted by using TRIZOL Reagent (GIBCO-BRL) according to the instructions provided by the manufacturer. Northern blot analysis was performed as previously described.

#### Measurement of [³H]Leucine Incorporation

VSMCs were serum-starved for 3 days and radiolabeled with [³H]leucine (2 μCi/mL, Amersham) for 2 hours. Cells were washed...
twice with ice-cold PBS and incubated with ice-cold 10% trichloroacetic acid for 30 minutes. After being washed with distilled water, the cells were lysed with 0.2N NaOH, neutralized with 0.2N HCl, and subjected to liquid scintillation counting.

Statistical Analysis
The values are mean±SEM. Statistical analyses were performed by ANOVA, followed by the Student-Newman-Keuls test. Differences with a value of P<0.05 were considered statistically significant. An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Ang II Induces DNA Binding Activity of MEF2A in VSMCs
We first examined whether Ang II induced the DNA binding activity of MEF2A. Ang II significantly increased the DNA binding activity of MEF2 in rat VSMCs (2.1±0.12-fold compared with control level, P<0.001; n=3) (Figure 1). The authenticity of the DNA-protein complexes was confirmed by cold competition experiments in which a 100× molar excess of double-stranded oligonucleotides encoding a wild-type or mutant MEF2 binding site was included in the reaction mixture and by use of a highly specific anti-MEF2A antibody,14 which supershifted the DNA-protein complexes. The Ang II–induced increase of MEF2A DNA binding activity was significantly suppressed when rat VSMCs were preincubated with 100 nmol/L Val (1.2±0.03-fold increase compared with control level, P<0.01 versus Ang II stimulation; n=3). In marked contrast, pretreatment with the p38 inhibitor SB or the calcineurin inhibitors FK and CyA did not remarkably inhibit the Ang II–induced increase of MEF2A DNA binding activity. We also confirmed that Ang II significantly increased the expression of MEF2A protein without affecting the expression levels of MEF2A transcripts (see online Figure 1).

Ang II–Induced Transcriptional Activation of c-jun Gene Is Mediated by MEF2- and p38-Dependent Pathways
To examine the roles of MEF2 in gene transcription in VSMCs, we used the human c-jun promoter because it has been reported that MEF2 is critically implicated in the transcriptional activation of the c-jun gene.23,24 Luciferase reporter constructs containing the wild-type human c-jun promoter (c-junwt promoter) or a mutant c-jun promoter (c-junmut promoter), in which the consensus MEF2 binding site was mutated, were transfected in VSMCs, and the cells were stimulated with Ang II. Ang II significantly increased the activity of the c-junwt promoter (Figure 2A). This increase was significantly suppressed by pretreatment with Val, whereas CyA or FK did not significantly inhibit the Ang II–induced increase of the c-junwt promoter activity. It has been suggested in several reports that MEF2CS387A, in which serine 387 of human MEF2C is replaced with alanine, works as a dominant-negative mutant of MEF2C.23,25 We found that serine 453 of human MEF2A is located in a similar alignment of amino acid sequences with serine 387 of human MEF2C. We substituted serine 453 with alanine (MEF2ASA) and examined whether it worked as a dominant-negative mutant. The Ang II–stimulated activation of the c-junwt promoter was significantly inhibited to the nonstimulated control level by cotransfection with MEF2ASA, suggesting that this mutant worked as a dominant-negative mutant and that Ang II–induced c-junwt promoter activation was mediated by the MEF2-dependent pathway. Because it has been reported that the p38-dependent phosphorylation and activation of MEF2 are implicated in the transcriptional activation of genes, especially the c-jun gene,23,24 we used dominant-negative and constitutively active mutants of MEK6, a kinase that activates p38. When MEK6AA was cotransfected with the reporter plasmid encoding the c-junwt promoter, Ang II–induced activation of the c-junwt promoter was suppressed to the control level, suggesting that the MEK6/p38-dependent pathway was involved in the Ang II–induced activation of the c-jun gene. In accordance with the result, SB significantly inhibited the Ang II–induced increase of the c-junwt promoter activity. Furthermore, MEK6EE significantly increased the activity of the c-junwt promoter in the absence of Ang II. In contrast, the constitutively active mutant of human calcineurin A, CalAΔC, did not promote the activation of the c-junwt promoter. We also used the c-junmut promoter. Ang II did not remarkably stimulate the activity of the c-junmut promoter. This result also supported our findings that Ang II stimulated the activity of the c-jun promoter via the MEF2-dependent pathway. Although MEK6EE stimulated the activity of the c-junmut promoter, the extent was smaller than that observed when the c-junwt promoter was used. CalAΔC did not stimulate the activity of the c-junmut promoter. Interestingly, forced expression of wild-type MEF2A decreased the activity of the c-junwt promoter, probably because MEF2A homodimers inhibited the formation of MEF2A heterodimers. A similar phenomenon was observed when MEF2C was
overexpressed along with a reporter plasmid encoding the wild-type c-jun promoter. 

Expression of the mutants used in these experiments was confirmed by Western blot analyses (see online Figure 2A). To confirm the involvement of MEF2A in the activation of the endogenous c-jun gene, we infected VSMCs with AdMEF2ASA and stimulated the cells with Ang II. Northern blot analysis showed that the Ang II–induced increase of c-jun gene expression was suppressed to the nonstimulated control level by infection with AdMEF2ASA (Figure 2B). The expression of AdMEF2ASA in VSMCs was confirmed by Western blot analysis (see online Figure 2B).

Figure 2. Ang II (ATII) stimulated the activity of the c-jun promoter via the MEF2- and p38-dependent pathways but not via the calcineurin-dependent pathway in VSMCs. A, VSMCs were transfected with 1.5 μg of reporter constructs encoding the wild-type human c-jun promoter or mutant c-jun promoter in which the consensus MEF2 binding site was mutated, along with 0.25 μg of pRL-TK. VSMCs were then stimulated with 10⁻⁷ mol/L Ang II for 48 hours, and cells were harvested for the luciferase assay. The total amounts of plasmid DNA transfected in VSMCs were adjusted by using the expression vector pcDNA3. SeaPansy luciferase activity was used as the internal control. The relative luciferase activity observed in control cells transfected with the wild-type c-jun promoter construct was calculated as 1.0, and the fold induction in each group is indicated. *P<0.001 vs control; #P<0.01 vs Ang II stimulation (n=6). B, VSMCs were infected with the indicated multiplicity of infection (MOI) of AdGFP or AdMEF2ASA when the culture medium was changed to low-serum medium. After 3 days, VSMCs were stimulated with 10⁻⁷ mol/L Ang II for 30 minutes, and 30 μg of total RNA was electrophoresed for Northern blot analysis. The membranes were hybridized with a 32P-labeled cDNA probe prepared from human c-jun cDNA. The membranes were then reprobed with a cDNA probe encoding GAPDH. Shown is a representative result of 2 independent experiments in which the same result was obtained.

Ang II Induces DNA Binding Activity of NFAT in VSMCs

We next examined whether Ang II stimulated the DNA binding activity of NFAT in VSMCs. Ang II significantly increased the formation of DNA-protein complexes (2.2±0.3-fold compared with control level, P<0.05; n=3) (Figure 3A). These DNA-protein complexes were abolished when the reaction mixture contained a 100× molar excess of the double-stranded oligonucleotide encoding the wild-type NFAT binding site but not when it contained a 100× molar excess of the oligonucleotide encoding the mutant NFAT binding site. The intensity of the bands corresponding to the DNA-protein complex was significantly reduced when rat VSMCs were pretreated with Val (1.1±0.1-fold increase compared with control level, P<0.05 versus Ang II stimulation; n=3), CyA (1.1±0.2-fold increase compared with control level, P<0.05 in VSMCs was confirmed by Western blot analysis (see online Figure 2B).
versus Ang II stimulation; n=3), or FK (1.1±0.1-fold increase compared with control level, P<0.05 versus Ang II stimulation; n=3), indicating that the Ang II–induced formation of the DNA-protein complexes was mediated by its type 1 receptor–dependent and calcineurin-dependent pathways. To examine more specifically whether the NFAT family of transcription factors was included in the complexes, we used an anti-NFAT3 antibody because it has been reported that NFAT3 is expressed in cardiac muscles, which, developmentally, have properties similar to those of VSMCs.15 We used human VSMCs in this case because the antibody used in the present study was designed to react specifically with human NFAT3. A single band corresponding to a DNA-protein complex was detected, and the density of the band was remarkably increased when human VSMCs were stimulated with Ang II (Figure 3B). The DNA–protein complex was competed away by a 100× molar excess of the wild-type double-stranded oligonucleotide but not by the mutant double-stranded oligonucleotide. Importantly, the intensity of the band corresponding to the DNA-protein complex was diminished when the reaction mixture contained the anti-NFAT3 antibody but not when it contained nonimmune serum. These results suggested that Ang II stimulated the DNA binding activity of the NFAT family of transcription factors and that at least NFAT3 was expressed in VSMCs.

Ang II Promotes Calcineurin/NFAT-Dependent Gene Activation in VSMCs
To examine whether Ang II induced the transcriptional activation of genes via the calcineurin/NFAT-dependent pathway in VSMCs, we prepared heterologous promoter constructs in which 3 copies of the wild-type (pGL2-3XNFATwt) or mutant (pGL2-3XNFATmut) NFAT binding site were subcloned upstream from the minimal essential promoter region of the murine c-fos gene (Figure 4A). Ang II significantly increased the promoter activity of pGL2-3XNFATwt (Figure 4B). This increase was significantly suppressed when VSMCs were pretreated with Val, CyA, or FK. Furthermore, CalAΔC significantly increased the activity of pGL2-3XNFATwt in the absence of Ang II. In marked contrast, Ang II did not significantly increase the activity of pGL2-3XNFATmut, nor did CalAΔC significantly increase the promoter activity of pGL2-3XNFATmut. These results suggested that Ang II promoted the NFAT-dependent transcriptional activation of genes via its type 1 receptor–dependent and calcineurin-dependent pathways in VSMCs.

Ang II Induces Transcriptional Activation of the NMMHCB Gene via the Calcineurin/NFAT-Dependent Pathway but Not via the MEF2-Dependent Pathway in VSMCs
We next examined whether Ang II stimulated the NMMHCB promoter activity and whether Ang II–induced activation of the NMMHCB gene was mediated by the MEF2- and/or calcineurin/NFAT-dependent pathways in VSMCs. Although the isolated human NMMHCB promoter region did not contain any consensus MEF2 or NFAT binding sites, Ang II significantly increased the activity of the NMMHCB promoter, and this increase was significantly suppressed to the control level by pretreatment with Val (Figure 5A). Ang II–induced activation of the NMMHCB promoter was partially but significantly inhibited by pretreatment with CyA and FK. In accordance with the results, CalAΔC significantly increased the activity of the NMMHCB promoter in the absence of Ang II. In contrast, the Ang II–induced increase of NMMHCB promoter activity was not significantly inhibited by pretreatment with SB or by cotransfection with MEF2ASA or MEF6AA. The results were further supported by the finding that overexpression of wild-type MEF2A or MEF6EE did not significantly increase the activity of the NMMHCB promoter. To further confirm the results, we infected VSMCs with AdMEF2ASA or AdCalAΔC and examined their effects. Ang II–induced expression of NMMHCB was remarkably suppressed by pretreatment with Val, CyA, or FK but not by infection with AdMEF2ASA (Figure 5B). Furthermore, AdCalAΔC infection increased NMMHCB expression in the absence of Ang II, and this increase was inhibited by CyA. The expression of AdCalAΔC

![Figure 4](http://circres.ahajournals.org/lehman/cirres-2002-000050F4/lehman-2002a.png)

Figure 4. Ang II (ATII) induced NFAT-dependent transcriptional activation of genes in VSMCs. A, Diagram of reporter constructs used in the experiments is shown. B, VSMCs were transfected with 2.0 μg of pGL2-3XNFATwt or pGL2-3XNFATmut, along with 0.25 μg of pRL-TK. VSMCs were then stimulated with 10⁻⁷ mol/L Ang II for 48 hours, and cells were harvested for the luciferase assay. The total amounts of plasmid DNA transfected in VSMCs were adjusted by using the expression vector pcDNA3. SeaPansy luciferase activity was used as the internal control. The relative luciferase activity observed in control cells transfected with the reporter construct encoding 3 copies of the wild-type NFAT binding site was calculated as 1.0, and the fold induction in each group is indicated. *P<0.001 vs control; #P<0.001 vs Ang II stimulation (n=6).
in VSMCs was confirmed by Western blot analysis (online Figure 2B). These results suggested that the Ang II–induced activation of the NMMHCB gene was, at least in part, mediated by the calcineurin/NFAT-dependent pathway but not by the MEF2- or p38-dependent pathway in VSMCs.

**Ang II Promotes Protein Synthesis via the MEF2-Dependent Pathway but Not the Calcineurin/NFAT-Dependent Pathway in VSMCs**

We finally examined whether the MEF2- and/or calcineurin/NFAT-dependent pathway was implicated in vascular hypertrophy (Figure 6). We infected VSMCs with AdMEF2ASA or AdCalAΔC and examined their effects on [3H]leucine uptake. Ang II significantly increased [3H]leucine incorporation, and this increase was significantly suppressed by pretreatment with Val but not with CyA or FK. AdMEF2ASA infection significantly suppressed Ang II–induced [3H]leucine uptake in a dose-dependent manner. AdCalAΔC infection did not significantly increase [3H]leucine uptake in the absence of Ang II. These results suggest that Ang II–induced [3H]leucine uptake is, at least in part, mediated by the MEF2-dependent pathway but not by the calcineurin/NFAT-dependent pathway.

**Discussion**

Although we have reported that MEF2A is a major isoform expressed in VSMCs and that its expression is upregulated when serum-starved VSMCs are restimulated with serum mitogens,14 the functions of MEF2 in VSMCs remain to be elucidated. Therefore, we examined whether MEF2 was implicated in Ang II–induced phenotypic modulation and/or hypertrophy of vascular myocytes. Ang II stimulated the DNA binding activity of MEF2A and its expression at the protein level without affecting the expression level of MEF2A transcripts (see online Figure 1), suggesting a posttranscriptional regulation of MEF2A expression in VSMCs. It has been found that although MEF2 transcripts are widely expressed in various tissues, the expression of the MEF2 protein and its transactivating function are found in more restricted tissues,7,8,26 suggesting a translational regulation of MEF2 expression. In fact, a translational repression by guest on April 5, 2017 http://circres.ahajournals.org/ Downloaded from
element was identified in the 3’ untranslated region of mouse MEF2A transcripts. Thus, our results are compatible with the previous finding that MEF2 expression is regulated at the translational level.

Ang II induced a transcriptional activation of the c-jun gene, and this increase was inhibited by MEF2ASA, MEK6AA, and SB but not by CyA or FK. The activity of the c-jun promoter was increased by cotransfection with MEK6EE in the absence of Ang II but not by cotransfection with CalAΔC. Furthermore, when a mutant of the c-jun promoter in which the consensus MEF2 binding site was mutated was used, the Ang II–induced increase of the mutant c-jun promoter activity was minimal, and MEK6EE–stimulated activation was smaller than that observed for the wild-type c-jun promoter. These results are compatible with previous findings that the activation of the c-jun gene is, at least in part, mediated by the MEF2 family of transcription factors and that p38-induced phosphorylation of MEF2 is involved in the transactivating function of MEF2. Although the Ang II–induced increase of MEF2A DNA binding activity was not affected by the p38 inhibitor SB, the Ang II–induced increase of c-jun promoter activity was significantly inhibited by SB and MEK6AA. A similar dissociation of the DNA binding activity and transactivating function of MEF2 has previously been reported. The calcineurin–mediated pathway stimulated the transactivating function of MEF2A without affecting its DNA binding activity in skeletal muscle cells. Thus, it appears that not only DNA binding of MEF2 but also posttranslational modifications such as phosphorylation or changes in its association with other nuclear factors are required for the MEF2-dependent transcriptional activation of genes.

Although it is well known that the calcineurin/NFAT–dependent pathway is implicated in the development of cardiac hypertrophy, the role of this pathway in VSMCs has not been clarified. We found that Ang II stimulated the DNA binding activity of NFAT and the NFAT-dependent transcriptional activation of genes, which were inhibited by CyA and FK. We also found that among the NFAT family of transcription factors, at least NFAT3 was expressed in VSMCs. It has been reported that NFAT1 (NFATp) and NFAT2 (NFATc) were expressed in VSMCs by using Western blot analysis and immunostaining, although the expression of NFAT3 was not examined in that study. Thus, several isoforms of the NFAT transcription factors may be expressed in VSMCs.

Although the promoter region of human NMMHCβ does not contain any canonical MEF2 or NFAT binding site, Ang II stimulated the NMMHCβ promoter activity, and this increase was partly mediated by the calcineurin/NFAT–dependent pathway but not by the MEF2– or p38–dependent pathway in VSMCs. Little is known about genes expressed in VSMCs whose promoter regions contain NFAT binding sites. However, the NFAT transcription factors, reportedly, do not always need to directly bind to DNA to induce a transcriptional activation of genes. NFATp enhanced the transcriptional activity of MEF2D by recruiting p300 without binding to DNA in T cells. Furthermore, it is known that genes activated during cardiac hypertrophy do not always possess NFAT binding sites in their promoters. Therefore, further studies are required to elucidate what kinds of genes are activated via the calcineurin/NFAT–dependent pathway during phenotypic modulation of VSMCs.

Surprisingly, Ang II–induced protein synthesis (hypertrophy) was mediated by the MEF2–dependent pathway but not by the calcineurin/NFAT–dependent pathway in VSMCs. It is of note that Ang II reportedly stimulates the production of reactive oxygen species (ROS) in VSMCs and that ROS and p38 are implicated in Ang II–induced protein synthesis. Therefore, it is possible that Ang II induces vascular hypertrophy via the ROS/p38/MEF2–dependent pathway. Collectively, our results indicate that MEF2 and calcineurin/NFAT have, at least partly, distinct roles in VSMCs. MEF2 appears to be implicated in vascular hypertrophy, whereas calcineurin/NFAT appears to be involved in phenotypic modulation, although both of them may be involved in the activation of the same genes in some cases.

In summary, Ang II induced the MEF2A– and calcineurin/NFAT–dependent transcriptional activation of genes in VSMCs. These pathways may be implicated in the pathological changes of VSMCs, such as dedifferentiation, hypertrophy, and hyperplasia, observed in atherosclerotic lesions. Modulation of these pathways may be a useful strategy in inhibiting the progression of atherosclerosis.

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Title: Angiotensin II Induces Myocyte Enhancer Factor 2- and Calcineurin/Nuclear Factors of Activated T Cells-Dependent Transcriptional Activation in Vascular Myocytes

MATERIALS AND METHODS

The amino terminally HA-tagged human MEF2A cDNA was prepared by polymerase chain reaction (PCR) using a human MEF2A cDNA clone previously isolated. The primers used for PCR were as follows: Sense primer; 5'-GAATTCGGGGGAAGAAATACAAACATACAC-3', antisense primer; 5'-CTCGAGTTAGGCACCCACGCCTCA-3'. The PCR primers were designed to amplify the coding region of human MEF2A from the second codon up to the stop codon. The amino terminally HA-tagged human mitogen-activated protein kinase kinase 6 (MEK6) and human calcineurin A 1-398 (CalAΔC), which lacks the carboxyl terminal calmodulin binding domain, were prepared by reverse transcription-PCR. PolyA RNA extracted from human heart (Clontech, Palo Alto, CA) was subjected to reverse transcription using a random primer. The cDNAs were used for subsequent PCR. The primers used for PCR were as follows: MEK6sense; 5'-GGATCCTCTCAGTCAAGGCAAGAAGCGA-3', MEK6antisense; 5'-CTCGAGTTAGGTCTCAGCTGTTGTACCCACGCTCA-3', CalAΔCsense; 5'-GGATCCATGTCGAGCCAAAGGCAATTG-3', CalAΔCanitise; 5'-CTCGAGTCAGTCTCCTTTATCACCTCCTTCCCGG-3'. The PCR-amplified products were subcloned in the pcDNA3HA vector. The primers used for mutagenesis were as follows: MEF2ASAprimer; 5'-CCCGACAGGAAATGGGGCGCggggCCCCTGTGGACAGTCTGAGCAGCTC-3', MEK6AA primer; 5'-CAGTGGCTACTTTGTTGGACGCTGTTGCTAAAAGCAATTTGACGACGCTGCTC-3', MEK6AAprimer; 5'-CAGTGGCTACTTTGTTGGACGCTGTTGCTAAAAGCAATTTGACGACGCTGCTC-3'.
CAGTGGCTACTTGGTGGACgaaGTTGCTAAAgaaaATTTGATGCAGGTTGCCAAACCA-3'. The lowercase, bold letters depict nucleotide substitutions to induce point mutations. Approximately 420 bp of the promoter region of the human c-jun gene was prepared by PCR. The primers used for PCR were as follows: c-junsense: 5'-CTCGAGCCGTCGACGGCGTGAAACTTGAG-3'; c-junantisense: 5'-AAGCTTTGAGCCCTTATCCAGCCCGAGCTC-3'. The PCR amplified product was digested with XhoI and HindIII, and ligated to the pGL2-basic vector (Promega, Madison, WI). The human c-jun promoter which contains a mutated MEF2-binding site was created by PCR. The primer used was 5'-AAGCTTTGAGCCCTTATCCAGCCCGAGCTCAGAATAGCCCATGATGTC-3'. The lowercase, bold letters indicate nucleotide substitutions to induce point mutations. The pGL2-basic vector which contains the minimal essential promoter region of mouse c-fos gene (pGL2basic-murine c-fos -50/+13) was prepared by ligating a double-strand oligonucleotide to the pGL2-basic vector at XhoI and HindIII sites. The sense-strand of the oligonucleotide was

TCGAATCCATTCACAGCGCTTCTATAAACGGCCAGCTGAGGCGCCTACTACTCCAACCGGCGACTGCA-3'.

Double-stranded oligonucleotides containing a wild type or a mutant NFAT binding site were then ligated to pGL2basic-murine c-fos -50/+13 at the SacI site which was located upstream of the minimal essential promoter region. The sense strands of the oligonucleotides were as follows: NFAT wild type; 5'-CTACATTGGAAAATTTTATTACACGAGCT-3', NFAT mutant; 5'-CTACATTGGcccaATTTTATTACACGAGCT-3'. These oligonucleotides were designed according to the sequences of human interleukin-2 promoter. Approximately 1.5 kb of the promoter region of human NMMHCB was prepared by PCR using human genomic DNA (Promega) as the template. The PCR primers were as follows: NMMHCBsense; 5'-CTCGAGCACAACCCCTTCTTTAGAGCTTCTGCC-3', NMMHCBantisense; 5'-AAGCTTCCGCTGCTTCCGCCGGCTCTTTA-3'. The PCR
products were digested with XhoI and HindIII, and ligated to the pGL2-basic vector. A fragment corresponding to the carboxyl terminal coding region of human c-jun was prepared by reverse transcription-PCR using total RNA extracted from human VSMCs. One µg of total RNA was subjected to reverse transcription using a random primer, and PCR was performed. The PCR primers used were as follows: c-jun sense primer; 5′-GCCGGTCTACGCAAACCTCAG-3′, c-jun antisense primer; 5′-GCACCACCTGTTAACGTGGTTC-3′. The PCR-amplified product was digested with BamHI and EcoRI, and subcloned in pBluescript SK(+) vector (Stratagene, La Jolla, CA). The insert was excised and used as a cDNA probe for Northern blot analysis. The full-length human MEF2A cDNA was also excised from the pcDNA3HA vector and used as a cDNA probe for Northern blot analysis. The nucleotide sequences of the all constructs were confirmed by cycle sequencing using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster, CA).

RESULTS

We examined whether ATII increased MEF2A protein expression in VSMCs. MEF2A was detected as two separate bands as we previously reported, probably reflecting differences in posttranslational status such as phosphorylation. When rat VSMCs were stimulated with 10^{-7}M ATII, expression of the MEF2A protein increased in a time-dependent manner (8 hrs poststimulation; 1.5±0.02 fold increase compared with control level, n=3, P<0.01) (online Figure 1A). ATII-induced increase of MEF2A expression was substantially inhibited when rat VSMCs were pretreated with 100 nM Val which was reported to be a highly specific antagonist for ATII type 1 receptors (Online Figure 1B), suggesting that ATII promoted MEF2A expression via its type 1 receptor. We also examined the expression of MEF2A transcripts. The results of Northern blot analysis showed a complex expression pattern of MEF2A transcripts. At least, three bands were detected. A similar expression pattern of the MEF2A transcripts was previously reported. The expression of MEF2A transcripts was not remarkably affected by ATII stimulation (Online Figure 1C). The result was compatible with our previous report that MEF2A expression was regulated at the
Expression of the mutants used in these experiments was confirmed by Western blot analyses in which anti-HA antibody was used to detect their expression, except for MEF2A constructs in which anti-MEF2A antibody was used, since the bands corresponding to MEF2A protein were hidden in background bands when anti-HA antibody was used (Online Figure 2A). Expression of AdMEF2ASA and AdCalAΔC in rat VSMCs was also confirmed by Western blot analysis using anti-HA antibody to detect CalAΔC and anti-MEF2A antibody to detect MEF2ASA (Online figure 2B).

REFERENCES


FIGURE LEGENDS

Online Figure 1 ATII induced MEF2A protein expression without affecting the expression of MEF2A transcripts in VSMCs. A) ATII stimulated MEF2A expression in a time-dependent manner. Rat VSMCs were serum-starved for three days and
stimulated with $10^{-7}$ M ATII for the indicated periods. One hundred $\mu$g of each protein extract was used for Western blot analysis using anti-MEF2A antibody. B) ATII-induced increase of MEF2A expression was inhibited by pretreatment with Val. VSMCs were serum-starved for three days and stimulated with $10^{-7}$ M ATII for 8 hrs in the presence and absence of 100 nM Val. Western blot analysis was performed in the same way as in A). C) ATII did not change the expression of MEF2A transcripts. Rat VSMCs were serum-starved for three days and stimulated with $10^{-7}$ M ATII for the indicated periods. Thirty $\mu$g of total RNA was loaded in each lane. The membranes were hybridized with a $^{32}$P-labeled cDNA probe prepared from human MEF2A full-length cDNA. The membranes were then reprobed with a cDNA probe encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Arrows indicate the positions of MEF2A transcripts. The relative intensity of each band corresponding to the top arrow is indicated at the bottom of the photograph.

**Online Figure 2**  
A) Expression of MEF2A, MEF2ASA, MEK6AA, MEK6EE and CalA$\Delta$C. The expression plasmids used in this study were transfected in COS7 cells, and 100 $\mu$g of each protein extract was used for Western blot analysis to confirm the expression of these mutants. Protein extracts prepared from non-transfected COS7 cells were used as the control (C). Anti-HA antibody was used to detect the expression of these mutants except for the MEF2A constructs in which anti-MEF2A antibody was used. B) Expression of AdMEF2ASA and AdCalA$\Delta$C in VSMCs. Twenty MOI of AdMEF2ASA or AdCalA$\Delta$C was used to infect rat VSMCs and 100 $\mu$g of each protein extract was used for Western blot analysis to confirm the expression of these viruses. Protein extracts prepared from non-infected rat VSMCs were used as the control (C). Anti-HA antibody was used to detect the expression of CalA$\Delta$C while anti-MEF2A antibody was used to detect MEF2ASA.